In vitro selection of other proteins than antibodies by means of ribosome display

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Abstract The in vitro selection and simultaneous evolution of proteins is feasible by means of ribosome display. Here, we describe the use of a protein bearing a binding property without being an antibody for affinity enrichment of the ternary complex, consisting of a protein, a ribosome and a encoding mRNA. The binding property was a simple affinity tag, namely Strep-tag II and His-tag. We could demonstrate that the selection of a specific mRNA encoding a shortened bovine heart fatty acid binding protein with a N-terminal His-tag was possible. After nine cycles of transcription, translation, affinity selection and reverse transcription PCR the protein with the His-tag could be enriched 10^8-fold.

Key words: In vitro translation; Selection of protein; Ribosome display; Affinity tag

1. Introduction

Nucleic acids with specific molecular recognition and catalytic properties have been identified from complex pools by SELEX (reviewed in [1]), since they are simultaneously genotype and phenotype. Nonetheless, the selection and directed evolution of proteins is of great interest, because they play an essential role in nearly all biological processes and are much more extensively used in diagnostics, therapeutic, and industrial applications. Most of the methods used for the selection of proteins as carrier of the phenotype have been based directly or indirectly on living cells. Examples of such approaches include phage display [2], plasmid display [3], and completely in vivo genetic approaches [4–6]. However, in vivo approaches are limited by transformation efficiency to 1–10×10^9 different molecules. This limitation can be overcome by using in vitro systems based on cell-free translation. Unfortunately, proteins are not genetic molecules and cannot be copied by any known enzymatic activity. Therefore, to construct an in vitro protein selection cycle, the amplifiable genomic information (mRNA) must be physically linked to the selectable information (protein). Two different approaches have been published which make such coupling possible. The mRNA–protein fusion technique is a covalent linkage between the 3′-end of the mRNA and the carboxyl terminus of the protein via puromycin [7]. The puromycin–mRNA fusion has to be repeatedly constructed for each selection cycle and is a time-consuming factor. The ribosome display is based on the possibility of expressing proteins from mRNA lacking a stop codon, and making direct use of the ternary complex, consisting of a protein, a ribosome and a encoding mRNA (PRM-complex), for affinity enrichment [8] (Fig. 1). One limitation of the ribosome display is that libraries must be screened under conditions in which the PRM-complex is stable (high Mg^{2+} and low temperature). All selections based on the ribosome display technique reported so far have been carried out with antibodies. The dissociation constant (k_d) of the selected binding affinities ranges from 0.04 to 10 nM. We wanted to find out if it is possible to extend this technique

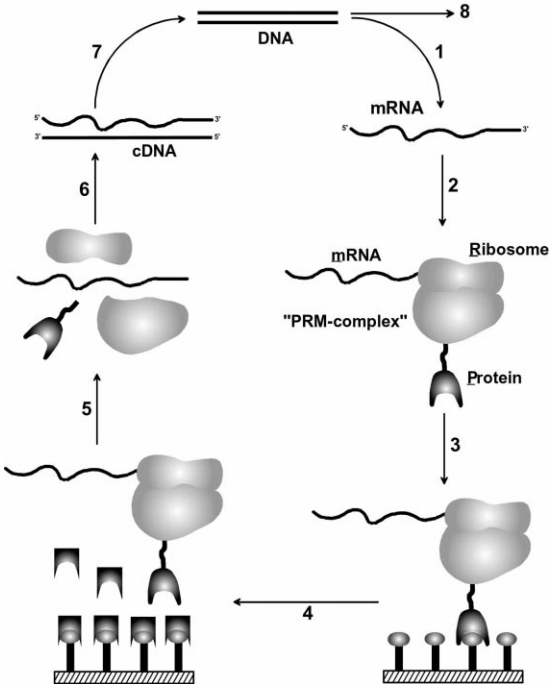

Fig. 1. Principle of in vitro ribosome display for screening protein libraries for ligand binding. (1) A DNA library is first transcribed into mRNA. (2) The mRNA lacking a stop codon is template for an in vitro translation. The formed PRM-complexes are stabilized by cooling on ice and increasing the magnesium concentration. (3) The desired PRM-complexes are affinity selected from the translation mixture by binding to the immobilized ligand. (4) The PRM-complexes can be eluted with a specific competitor. (5) Dissociation of the complexes by adding EDTA. (6) RT of the isolated mRNA. (7) Amplification of the cDNA by PCR, whereby a T7 promoter is introduced. This DNA is then used for the next cycle of enrichment and (8) can be analyzed by cloning and sequencing.

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to other proteins than antibodies, i.e. proteins possessing other types of affinities. Furthermore, the knowledge about the k_d value essential for selecting PRM-complexes is of great interest. Here, we report the establishment of the ribosome display technique with a protein carrying an affinity tag.

2. Materials and methods

2.1. Construction of plasmids

Standard methods for molecular biology were used [9]. The plasmids pHMFA and pFA+NStII [10] were the starting point for further constructs. Both have a T7 promoter, a sequence for the 5′ untranslated region of phage T7 gene 10 followed by a coding sequence for FABP and a T7 transcription terminator 150 bp downstream of the coding sequence. The pFA+NStII additionally has 30 bp encoding a linker and the Strep-tag II [11] at the N-terminus of the protein. The two vectors were digested with *Pst*I/*Bam*HI and the hybridized oligonucleotides lpp-for (5′-GCTGACCCGTTTAGAGGCCCCAAGGGGTTATGGAATTCACCTTTAAGCAGCTCG-3′) and lpp-rev (5′-GATCCGAGCTGCTTAAAGGTGAATTCCATAACCCCTTGGGGCCTCTAAACGGGTCAGCTGCA-3′) were cloned into them. In the cause of this process, a modified lipoprotein terminator (lpp term) was introduced at the C-terminus of the protein and at the same time, the stop codon was removed. The resulting plasmids were termed pFAlpp and pFAlpp+NStII.

Another construct designated pFAlpp+NHis was prepared by PCR from pFAlpp using the following primer pairs: FAF1 (5′-CGCGCGCCATGGTGCATCATCATCATCATCATGTGGACGCCTTCGTGGGTACC-3′) and FAR1 (5′-GAAGAGCAGTCAGTGGGAGGAGAGGGCAGGTCATGCCTGTTT-3′), which introduces a single valine followed by a hexahistidyl-tag at the N-terminus of the protein.

2.2. In vitro transcription

The mRNA transcripts were obtained by in vitro transcription using PCR products or *Bam*HI-treated plasmids with T7 RNA polymerase (Stratagene) following the protocol of Triana-Alonso et al. [12] with minor modifications.

The reaction mixtures were incubated for 2 h at 37°C and contained 80 mM HEPES·KOH (pH 7.5), 22 mM $MgCl_2$, 1 mM spermidine, 20 nM plasmid DNA or 100 nM PCR product, 10 mM DTE, 120 μg/ml BSA, 0.4 U/μl RNase inhibitor (Promega), 5 U/ml inorganic phosphatase (Sigma), 1 U/μl T7 RNA polymerase (Stratagene), 3.75 mM ATP, CTP, GTP, and UTP. For the radiolabeling of mRNAs the concentration of CTP was 2 mM and [α-^{35}S]CTP (Amersham) with a molar activity of >1000 Ci/mmol was added to a final concentration of 0.2 μCi/μl. After in vitro transcription the reaction mixture was treated with 0.2 U/μl RNase-free DNase I (Roche) for 15 min at 37°C. The transcripts were purified by phenol extraction followed by gel filtration with a NAP5 column (Pharmacia) and precipitated with isopropanol. The pellets were resolved in water. Transcripts were analyzed by agarose gel electrophoresis.

2.3. In vitro translation

The in vitro translation reaction is based on an *Escherichia coli* S30 lysate (strain D10) and was performed as described [13] with several modifications. Translation was carried out for 8 min at 37°C in a 250 μl reaction mixture that contained the following components: 50 mM HEPES·KOH (pH 7.6), 70 mM KOAc, 30 mM NH_4Cl, 14 mM $MgCl_2$, 0.1 mM EDTA, 0.02% NaN_3, 0.15 mM leucine, 0.4 mM each amino acid (leucine omitted), 1 mM each of ATP and GTP, 0.5 mM each of CTP and UTP, 30 mM phosphoenolpyruvate, 10 mM acetyl phosphate [14], 4% polyethylene glycol 2000, 20 μg/ml rifampicin, 0.1 mg/ml total *E. coli* tRNA, 100 μM folinic acid, 100 U/ml RNase inhibitor (Promega), 26% (v/v) S30, 0.2–0.6 μM mRNA and 5 μM anti-ssrA oligonucleotide [8]. 0.2 μM L-[^{14}C]leucine (25 dpm/pmol; Amersham) was added instead of 0.15 mM leucine for labeling the synthesized proteins.

2.4. Analysis of the synthesized protein

The incorporation of L-[^{14}C]leucine into the synthesized proteins was determined by liquid scintillation counting of the trichloroacetic acid-insoluble material as described [15]. The reaction products were also analyzed by SDS–PAGE [16] followed by an autoradiography in a 'Phosphoimager' system (Molecular Dynamics).

2.5. Affinity selection of PRM-complexes and isolation of mRNA

The translation was stopped by cooling on ice, and adding 1 M $Mg(OAc)_2$ to a final concentration of 50 mM [8] and dilution buffer (50 mM Tris·HOAc, pH 7.5/150 mM NaCl/50 mM $Mg(OAc)_2$/0.1% Tween 20) to a final volume of 625 μl. The samples were centrifuged for 5 min at 4°C at $15\,000\times g$ to remove insoluble components. The supernatant was applied to a 250-μl Ni^{2+}–iminodiacetic acid (IDA)–agarose (Novagen) column. After six washes with 500 μl ice-cold washing buffer (50 mM Tris·HOAc, pH 7.5/150 mM NaCl/50 mM $Mg(OAc)_2$/5–10 mM imidazole) the retained PRM-complexes were eluted with 500 μl ice-cold elution buffer (washing buffer with 300 mM imidazole). The released PRM-complexes were treated with 0.5 M EDTA until the final concentration was 50 mM and were precipitated with isopropanol in the presence of 0.3 M NaOAc (pH 5.5) and 40 μg glycogen. The 'pellet' was dissolved in 30 μl water and used for reverse transcription (RT).

2.6. Analysis of recovered mRNA after affinity selection

[α-^{35}S]CTP-labeled mRNAs were used to examine the integrity and amount of mRNA. For electrophoretic analysis 20 μl of a translation reaction or comparable amounts of other fractions were incubated for 30 min at 37°C with 20 mM EDTA, 0.5% SDS and 0.1 g/l proteinase K. The mRNA was purified by isopropanol precipitation and separated by using a 6% polyacrylamide gel containing 7 M urea, 0.1% SDS and TBE followed by an autoradiography in a 'Phosphoimager' system (Molecular Dynamics).

For TCA precipitation two identical samples of every fraction were precipitated with 5% TCA [9] and quantified by scintillation counting.

2.7. RT-PCR

Reverse transcription was performed using Superscript II reverse transcriptase (Gibco BRL) according to the supplier's recommendation with the following primer (5′-CCCCTTGGGGCCTCTAAACGGGTCAGCTGCAGTGCCGTGGGTGAGTGTCAGAATGAGTTTCCCGTCAACCATCTCCCG-3′). 30 cycles of PCR were performed using *Taq* DNA polymerase (Gibco BRL) according to the supplier's recommendation using the following primer pair: T7F (5′-GAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAG-3′), which introduces the T7 promoter, and lppR (5′-GGATCCGAGCTGCTTAAAGGTGAATTCCATAACCCCTTGGGGCCTCTAAACGGG-3′), which introduces the lpp-term. PCR products were analyzed by agarose gel electrophoresis, purified from the gel and directly used for transcription.

3. Results

3.1. Design of mRNAs and their corresponding PCR products

The used mRNAs bear the untranslated region of phage T7 gene 10, which encodes a stem–loop structure at the beginning of the mRNA, followed by the coding sequence for FABP. The sequence encoding the last 10 amino acids of FABP and the stop codon were replaced by the lipoprotein terminator of *E. coli*, which encodes a 3′ stem–loop at the RNA level. Therefore, the mRNA is protected against exonucleases at both ends [8]. The mRNAs obtained by in vitro transcription from pFAlpp, pFAlpp+NHis and pFAlpp+NStII or the corresponding PCR products were designated mFA, mFAHis and mFAStII, respectively (Fig. 2).

3.2. Formation of PRM-complexes

We started to investigate the formation of PRM-complexes by translation of mFAStII followed by ultra-centrifugation (90 min, $250\,000\times g$, 4°C) on the assumption of collecting the PRM-complexes in the pellet and thus separating them from the mixture. An identical mRNA with stop codon served as a control. Directly after the translation the PRM-complexes were stabilized by adding $Mg(OAc)_2$ to a final concen-

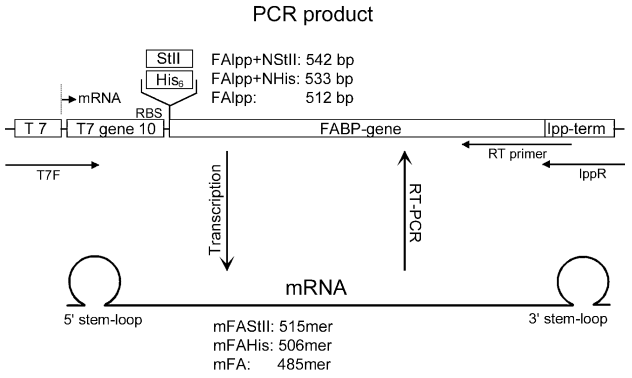

Fig. 2. Schematic drawing of a PCR product and its corresponding mRNA used for ribosome display. T7 denotes the T7 promoter, RBS the ribosome binding site, which is part of the untranslated region of phage T7 gene *10*. StII and His_6 indicate the presence of the Strep-tag II- and His-tag sequence, respectively. The location of the primers for RT-PCR is shown.

tration of 50 mM and cooling on ice. The amount of synthesized and therefore radioactively labeled protein was measured in the supernatant and the pellet after ultra-centrifugation as described. In the approach with mFAStII, nearly 60% of the synthesized protein were found in the pellet, whereas only about 33% were detected when using the identical mRNA with stop codon. On the basis of this experiment with ultra-centrifugation we ascertained that the translation lasted 15 min and the amount of anti-ssrA oligonucleotide was 5 μM.

3.3. Affinity isolation of PRM-complexes

The isolation of PRM-complexes with the help of the binding affinity from the protein portion was tested. Therefore, the amount of radiolabeled mRNA was measured after affinity chromatography by TCA precipitation. We tried the Strep-tag II and the affinity matrix StrepTactin Sepharose [17], since the purification of in vitro synthesized proteins works very well with this system (to be published). The PRM-complexes from a translation reaction with mFAStII were subjected to a modified Strep-tag II affinity chromatography (4°C, buffers with 50 mM $Mg(OAc)_2$). The amount of eluted mRNA was not significantly higher than the control reaction with mFAlpp and thus without Strep-tag II.

An in vitro translation with mFAStII under the new conditions was carried out to ensure that the presence of the PRM-complex is the reason for the non-existing binding capacity of the protein. One half of the reaction mixture was treated with $Mg(OAc)_2$ and cooled on ice to stabilize the PRM-complexes. The other half was incubated at 37°C for 10 min after adding puromycin to a final concentration of 5 mM, then also treated with $Mg(OAc)_2$ and cooled on ice. Puromycin is an antibiotic that mimics the aminoacyl end of Tyr-tRNA and causes the termination of polypeptide chain synthesis by entering the ribosomal A site and accepting the nascent peptide as a result of the peptidyl transferase activity of the ribosome [18]. The two samples were tested by ultra-centrifugation and affinity chromatography (Fig. 3). The synthesized protein was radioactively labeled and therefore detectable after TCA precipitation and SDS–PAGE. As expected, the ultra-centrifugation revealed that the amount of protein in the pellet was decreased from 75% to 13% after adding puromycin, whereas it increased in the supernatant as calculated by TCA precipitation. 22% of the labeled protein from the sample treated with puromycin was eluted after affinity chromatography and only 5% of the other sample where the PRM-complex was stabilized. This result shows that only the free protein has the potential for binding its affinity ligand instead of the protein as part of the complex. All the achieved results are only an indirect proof of the existence of the PRM-complex, but its influence on the loss of binding to the affinity matrix seems to be obvious. We think that the binding affinity of the Strep-tag II is not sufficient for isolating the whole PRM-complex. The Strep-tag II was developed to purify proteins in the native state under very mild buffer conditions due to the fact that its k_d is only 1 μM [17]. The achieved results with the Strep-tag system for purification of in vitro synthesized proteins are very satisfying, but the isolation of the PRM-complex with approximately 2700 kDa requires a stronger binding affinity.

One of the most widely used tags for protein purification with a strong binding affinity is the hexahistidyl tag (His-tag). Its imidazole moieties can chelate the free coordination sites of Ni^{2+} ions which are themselves immobilized as chelate complexes of IDA or nitrilotriacetic acid (NTA) bound to a solid support [19]. An in vitro translation with 0.1 μM radiolabeled mFAHis followed by affinity isolation of the PRM-complex on Ni^{2+}–IDA–agarose showed that more than 3% of the mRNA were eluted. On the other hand, the control reaction with mFA showed about 1.4% mRNA in the elution fraction. This result revealed that basically, the isolation of

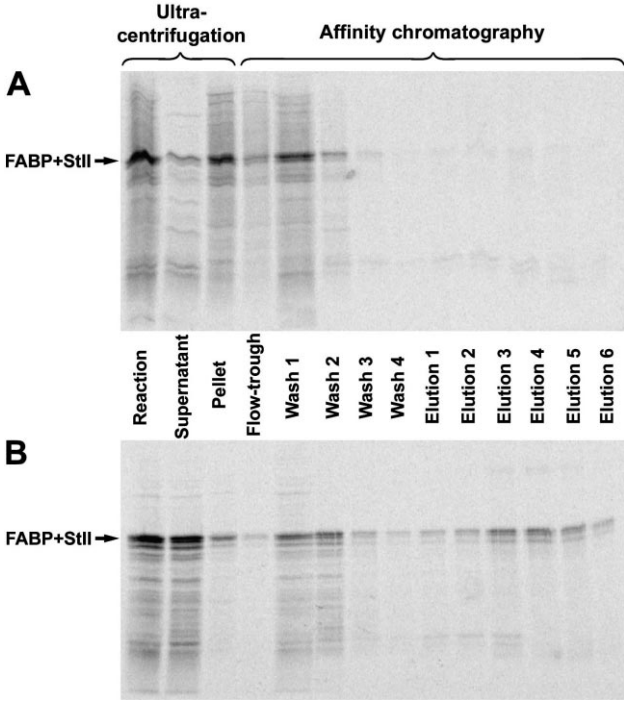

Fig. 3. Influence of the PRM-complex to the binding capacity of the Strep-tag II. An in vitro translation system was used to synthesize FABP+StII and form the corresponding PRM-complex. The translation mixture was divided and subjected to an ultra-centrifugation and an affinity chromatography. The translation products were separated by SDS–PAGE. Shown is the autoradiogram. A: Behavior of the intact PRM-complex. B: Behavior of the released protein after addition of puromycin.

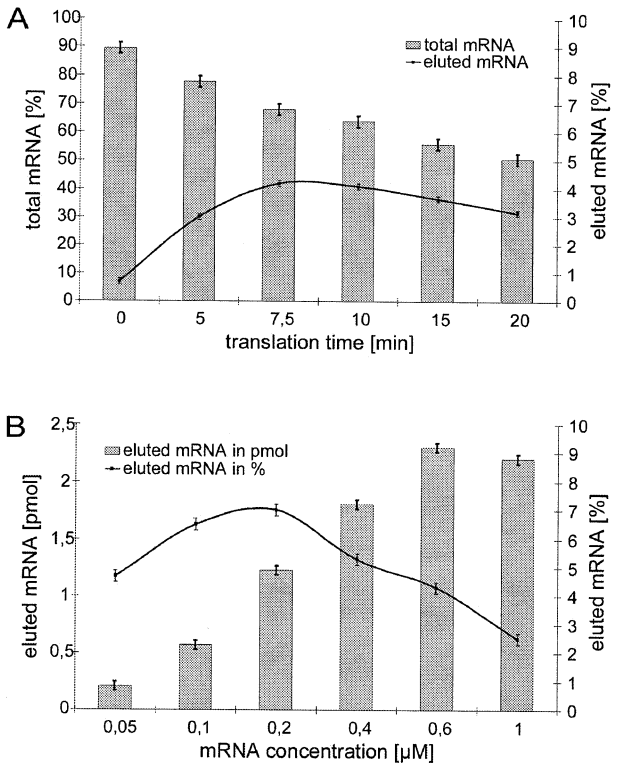

Fig. 4. Effect of different parameters on the amount of eluted mRNA after one cycle of ribosome display. A: Influence of the translation time. Total mRNA denotes the amount of mRNA after incubation and eluted mRNA after one cycle of ribosome display in relation to the amount of mRNA before incubation. B: Influence of the mRNA concentration. Eluted mRNA in percent denotes the amount of mRNA in relation to the amount of used mRNA and eluted mRNA in pmol the absolute amount of recovered mRNA.

the PRM-complex by means of His-tag affinity chromatography is achievable.

3.4. Optimizing the yield of isolated PRM-complexes

Different parameters influencing the yield of eluted radiolabeled mRNA as indirect proof for the PRM-complexes were tested on the basis of the His-tag system using mFAHis and mFA as control.

Firstly, the translation time of a 100-μl reaction containing 200 nM mRNA was investigated. The translations were performed at 37°C for 0, 5, 7.5, 10, 15 and 20 min. The total amount of mRNA after translation decreased continuously with increasing incubation time. The amount of eluted mRNA rose up to 4.2% after 7.5 min and slightly decreased with further incubation (Fig. 4A).

Secondly, the influence of ribonucleoside–vanadyl complex (RVC), which is an efficient RNase inhibitor [20], was examined. In our system, up to 0.5 mM of RVC can be used without inhibiting the translation reaction. The use of RVC during translation led only to a slight increase of total mRNA after incubation, but the amount of eluted mRNA was always decreased. The best results were achieved without RVC (data not shown).

Thirdly, we tried to reveal the influence of the mRNA concentration. Therefore, 100 μl translation reactions containing 0.05, 0.1, 0.2, 0.4, 0.6 and 1 μM mFAHis were incubated for 8 min at 37°C. The relation between eluted mRNA to used mRNA was the most favorable at 0.2 μM, but the highest number of mRNA molecules was recovered at 0.6 μM (Fig. 4B).

Finally, the conditions of the chromatography were optimized in order to decrease the background and increase the recovered mRNA. The sample was diluted with the so-called buffer to 2.5× translation volume after adding $Mg(OAc)_2$. The washing buffer for the washing steps 1–4 contained 5 mM imidazole and for the washing steps 5 and 6, 10 mM.

The combination of these results led to a low level of unspecific binding to the Ni^{2+}–IDA–agarose and increased the yield of mFAHis after one round of affinity selection up to 8% of input mRNA as calculated by TCA precipitation of radiolabeled mRNA (Fig. 5). The integrity of the mRNA was analyzed by denaturing polyacrylamide gel electrophoresis followed by autoradiography. As a result the amount of full length mRNA was 5% and the background of the system with mFA as template reached about 0.3%.

3.5. Specific enrichment of a desired mRNA through multiple rounds of selection

Before we started the test selection, identical amounts of the two mRNAs were mixed and used for RT-PCR, followed by transcription and another RT-PCR. None of them was favored in the RT-PCR or the transcription (data not shown). It is a fundamental demand that the amplification between the two species does not occur on the level of PCR.

Furthermore, different elution strategies for recovering the mRNA were examined (Fig. 6). The elution of the PRM-complex with a specific competitor or a competing ligand are useful, particularly when a special binding property has to be selected. The mRNA can be recovered from the complexes after adding EDTA or phenol extraction. The dissociation of the ternary complexes with EDTA or the direct RT-PCR [21] are also possible. These methods allow selection for high af-

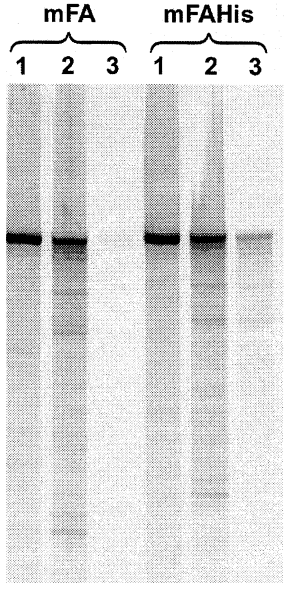

Fig. 5. Analysis of mRNA during one cycle of ribosome display. The radiolabeled mRNAs were isolated from the reaction mixtures and the PRM-complexes before and after Ni^{2+}–IMAC. (1) Reaction mixture, (2) reaction mixture after in vitro translation, (3) eluted PRM-complexes. The mRNAs were separated on a 5% denaturing polyacrylamide gel. Shown is the autoradiogram of the PAGE.

Table 1
Number of selection cycles necessary for enrichment of the diluted mRNA

Ratio of mFAHis:mFA	Number of cycles necessary for selection
$1:10^2$	3
$1:10^4$	4
$1:10^5$	5
$1:10^8$	9

mFA and mFAHis were mixed at different ratios and used for ribosome display.

finity and even covalent binders. Since we are only interested in eluting specific binders and our system is based on Ni^{2+}–IDA–agarose the use of EDTA is excluded during the selection step. Afterwards a reasonable combination of the possibilities of recovering selected mRNA molecules might be effective. Our optimized strategy combines the EDTA dissociation and the direct RT. The eluted PRM-complexes were firstly treated with EDTA, secondly precipitated with isopropanol and 10 μl of the dissolved pellet were finally used for RT. The RT primer was designed to hybridize upstream of the 3′ end of the mRNA which is possibly still covered by the ribosome. The 3′-end of the mRNA was regenerated during the PCR.

A mixture of two mRNAs served as a starting pool for a selection. The goal of the selection was to bind on Ni^{2+}–IDA–agarose. Using ribosome display a mRNA encoding a His-tag should be enriched. Therefore, mFA and mFAHis were mixed in different ratios and used for ribosome display. Their PCR products differ slightly in length (21 bp), because of the existence of the His-tag and a linker codon, and can thus be distinguished after agarose gel electrophoresis. Depending on the ratio of dilution different numbers of cycles according to Fig. 1 were necessary to enrich the PCR product encoding FABP with His-tag (Table 1). Even when using a ratio of $1:10^8$ (one mFAHis in relation to 10 000 000 mFA-molecules) 'one molecule' could be selected after nine cycles (Fig. 7). The PCR products which went through nine cycles of selection were cloned and analyzed. Eight out of 10 sequenced clones had the full His-tag sequence, and the remaining two had a shortened His-tag sequences encoding five and four histidines, respectively (data not shown). The sequence analysis showed that the clones contained between 5 and 13 base changes. At the protein level, the selected clones carried between 0 and

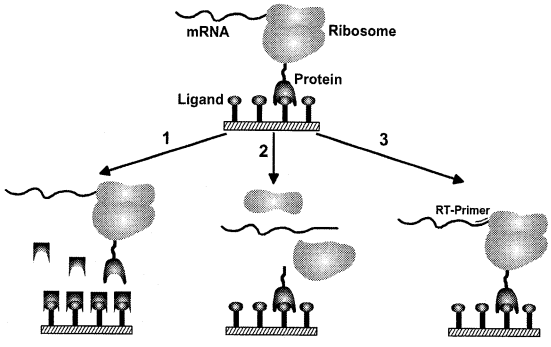

Fig. 6. Different elution strategies for recovering the mRNA from the selected PRM-complex. (1) The selected PRM-complex can be eluted with a specific competitor and the mRNA can be recovered after disruption of the complex. (2) The dissociation of the PRM-complex by adding EDTA or (3) the direct RT followed by PCR are possible.

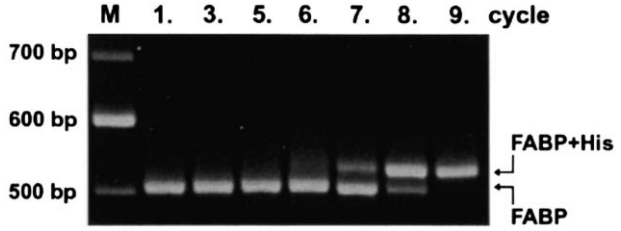

Fig. 7. Enrichment of FABP+His from a mixture with FABP by ribosome display. The mRNA of FABP+His was mixed with the mRNA of FABP at a ratio of $1:10^8$ and used for ribosome display. After affinity selection of PRM-complexes carrying a His-tag, the mRNA was amplified by RT-PCR and analyzed by agarose gel electrophoresis. Lane M is a 100-bp DNA ladder. The other lanes show the PCR products after different selection cycles.

5 exchanged amino acids. The error rate of the PCR and therefore the diversification of the library can be increased by error prone PCR [22] or by using a low fidelity polymerase, but can also be decreased by the use of a proof-reading polymerase.

4. Discussion

The results demonstrate that even the 10^8-fold enrichment was successful. With our model system the selection of a specific mRNA via the accompanying protein property was shown. This study describes for the first time the use of a protein possessing a binding property without being an antibody for ribosome display. This stands in contrast to previous works which carried out phenotypic selection for ligand binding with antibodies. Another new feature we managed to introduce was the use of an affinity tag for isolating PRM-complexes, thus establishing the ribosome display technique. On the basis of our results it should be possible to do in vitro evolution of proteins or just a special portion of them which are optimized for other reasons than recognition and binding target molecules. Since ribosome display is a monovalent system the high binding affinities in the low nanomolar or even picomolar range selected so far are not always desired but rather necessary for selecting the whole complex. Therefore, the selection of proteins with affinities in the micromolar range is, in contrast to the multivalent phage and plasmid display systems, not achievable. All selection steps of ribosome display are carried out in vitro; thus it is possible to overcome the limitations of in vivo approaches. The library size that can be handled correlates directly with the number of active ribosomes in the translation mixture. The concentration of ribosomes in our system is about 1 μM and even if only 20% of them are active it should be possible to screen libraries of $\sim 10^{14}$ independent members.

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